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Replication of Rat Coronaviruses in Intestinal Cell Line, RCN-9, Derived from F344 Rats

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Abstract: *To examine the susceptibility of the epithelial cell line to rat coronavirus (RCV), we inoculated sialodacryoadenitis virus and Parker's RCV into five cell lines; JTC-19, rat L2, LLC, RCN-9 and LBC cells originating in the lungs, intestines and mammary tumors of rodents. Both RCVs were replicated in LBC and RCN-9 cells, but not in the others. The infectivity titers of both RCVs grown in RCN-9 cells were significantly higher than those in LBC cells in every passage (2.5–3.9 log rate). Both RCVs replicated in LBC cells showed higher tropism to RCN-9 cells than to LBC cells, suggesting that RCN-9 cells are more suitable for the replication of RCVs than LBC cells. The RCN-9 cell line would be useful for the investigation of RCV infection in rodents.*

Key words: *Rat coronavirus, RCN-9 cell, SDAV*

Many laboratory animal facilities pay close attention to mouse coronavirus infections in transgenic and gene-targeting mice [16], since recent findings suggest that mouse hepatitis virus (MHV)-infected mice show an altered immunological state [3, 13] and act as a source of infection to immunodeficient roommates. In contrast to MHV in mice, the apprehension for rat coronavirus (RCVs) infection, including sialodacryoadenitis virus (SDAV) and Parker's rat coronavirus (PRCV) in laboratory rats and mice, has not been greatly emphasized, although it has been shown that infant mice are susceptible to SDAV [1] and that infant rats are susceptible to MHV [14] by intranasal inoculation. Furthermore, intranasal inoculation of SDAV into female rat results in reproductive failure, inducing disorder in estrus cycles and fetal death [9, 15].

Several cells are susceptible to RCVs infection including primary rat kidney cells [2], 3T3 cells [10], mouse L-2 cell line [11, 12], a subline of L-929 fibroblast cells, and LBC cells [6, 7] from rat mammary tumor. No replication or remarkable CPE of RCV in rat cell lines derived from lungs and intestines have been reported. We searched for rodent epithelial cell lines with high susceptibility (virus yield) to RCV to study the relation between RCVs infection and epithelial cells of enteric canal.

We inoculated RCV into five cell lines, JTC-19, L2

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Table 1. Characteristics of the five cell lines inoculated with RCVs

Cells	Obtained Tissue	from derived from	Characteristics
JTC-19	RCB[1]	rat lung	fibroblast-like
L2	ATCC[2]	rat lung	?
LLC		RCB mouse lung	Lewis lung carcinoma
RCN-9	RCB	F344 rat colon	epithelial-like
LBC[3]		Lewis rat mammary gland	adenocarcinoma

[1] Riken Cell Bank of Japan. [2] American Type Culture Collection. [3] LBC cells were kindly provided by Dr. N. Hirano, of Iwate University, Japan.

(different from mouse L-2), LLC, RCN-9, and LBC. The origins and characteristics of these cell lines are summarized in Table 1. We selected four cell lines (JTC-19, L2, LLC, and RCN-9), originating in rodent lungs and intestines, because the primary targets of many strains of MHV are the respiratory and intestinal tracts, and we assumed that the replication of SDAV and PRCV may also lie in the cell lines originating in these tracts. LBC cells were investigated to compare with the others. LBC cell cultures were grown and maintained in minimum essential medium (MEM) (Gibco Lab., NY, U.S.A.) and the other cells were grown and maintained in RPMI-1640 (Nissui Co., Tokyo). Both media were supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone Lab., UT, U.S.A.), 100 IU/m*l* penicillin, 100 μg/m*l* streptomycin, and 0.5 μg/m*l* amphotericin B. The cells were propagated in 75 cm^2 plastic flasks (Costar Co., MA, U.S.A.) at 33°C and at 37°C in a humidified atmosphere containing 5% CO_2.

At 4 days post infection (dpi) with the original LBC-adapted viruses, SDAV (Yale strain #681; SDAV-681) and PRCV kindly provided by Dr. N. Hirano, Iwate University of Japan, and then centrifuged at 2,000 rpm for 10 min at 4°C. These initial infectious fluids were frozen and thawed three times, and RCV/LBC/0 were then inoculated into the five cell lines.

For the indirect immunofluorescence assay (IFA), virus-antigen slides for the five cell lines were prepared on 4 dpi. The cells were mounted on a spot glass slide and fixed in cold acetone, then incubated with rat anti-SDAV and anti-PRCV antisera obtained from the American Committee on Laboratory Animal Diseases that were 4-fold diluted from 1:100 to 1:25,600 with cation-free 10 mM phosphate-buffered saline (PBS).

Table 2. Infectivity titers of RCVs with several passage levels in the two cells

Sup.[2]	Infectivity titers[1] in RCN-9[3]	in LBC[4]	R/L log ratio[5]
SDAV-681/			
LBC/0	7.6×10^5	3.4×10^4	1.3
LBC/1	2.2×10^5	1.0×10^3	2.3
RCN-9/1	1.6×10^7	5.5×10^3	3.5
RCN-9/2	1.0×10^{10}	1.2×10^6	3.9
PRCV/			
LBC/0	2.2×10^3	5.5×10^1	1.6
LBC/1	4.0×10^3	1.0×10^3	0.6
RCN-9/1	2.2×10^7	4.0×10^3	3.7
RCN-9/2	3.4×10^8	1.2×10^6	2.5

[1] Titers are shown in FFU/m*l*. [2] Virus strain/propagated cell/passage No. [3] Cultured in RPMI-1640 at 37°C. [4] Cultured in MEM at 33°C. [5] Log ratio of infectivity titer in RCN-9 cells to titer in LBC cells.

The cells were visualized with fluorescein isothiocyanate-conjugated goat anti-rat IgG (Cappel Co., PA, U.S.A.) diluted at 1:20 in PBS, then observed by fluorescence microscopy. Both RCVs replicated in LBC and RCN-9 cells, but no immunofluorescence positive cells were observed in the other cell lines. The IFA titers of standard antisera for SDAV-681 and for PRCV in both LBC and RCN-9 cells exceeded 1:6,400.

One hundredth of the fluid of the former RCV passages in the LBC and RCN-9 cells was inoculated into fresh confluent cells. The first and/or second RCV infectious fluids were collected from the LBC cells at 4 dpi and from the RCN-9 cells at 2 dpi, and were named RCV/LBC/1 and RCV/RCN-9/1, 2 (Table 2). The virus infectivity of these RCV fluids was detected by counting the foci visualized by an ABC method. In brief, monolayers of LBC and RCN-9 cells in 12-well

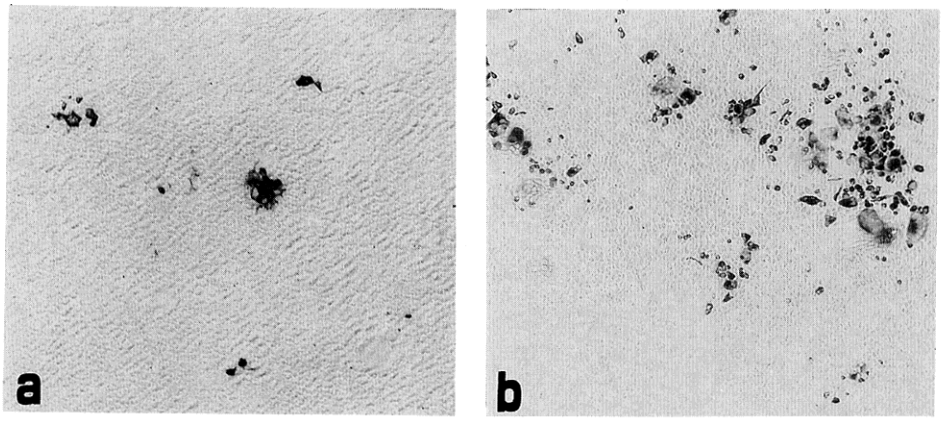

Fig. 1. Micrograph of SDAV-681 infected LBC cells on 4 dpi (a) and RCN-9 cells on 2 dpi (b). Initial SDAV-681 virus fluid (/LBC/0) was inoculated into both cells ($1:10^3$ dilution). In LBC cells (a), small foci without CPE are stained darkly by the ABC method. Large foci are observed in RCN-9 cells (b). ×64.

plastic plates (Costar Co., MA, U.S.A.) were infected with a 10-fold dilution of the virus. After virus adsorption for 2 hr at 37°C, the LBC cells overlaid with the maintenance medium, including 0.6% agarose (FMC Co., ME, U.S.A.), were incubated for 2 days at 33°C. Similarly RCN-9 cells were overlaid and incubated at 37°C. The gel media were then removed and the cells were washed, fixed with 4% paraformaldehyde in PBS, and rinsed with PBS supplemented with 0.3% Triton X-100 (PBST). They were incubated with above the rat antisera diluted at 1:5,000 in PBST, followed by incubation with biotinylated anti-rat IgG (1:500; Vector Inc., CA, U.S.A.), and finally incubation with avidin/biotinylated horseradish peroxidase complex (ABC). After each reaction, the cells were rinsed for 30–60 min in two changes of PBST. A dark purple color was developed by incubation for 30 min with PBS containing 0.02% 3.3'-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto), 0.006% H_2O_2, and 0.4% nickel ammonium sulfate. The visualized foci consisting of more than 2 cells were counted under a dissecting microscope (Figs. 1a and 1b). Although the immunoreactions of the foci in LBC cells were more intense than those in RCN-9 cells, the foci in LBC cells were smaller than those in RCN-9 cells. This finding suggests that the RCV grown in RCN-9 cells may more easily infect adjoining cells than the RCV grown in LBC cells.

Numerous syncytial giant cells, showing CPE and confluent vesicular degeneration, were observed in RCV-infected RCN-9 cells on and after 2 dpi, and extremely giant cells were induced by SDAV-681 (Figs. 2a and 2b). No syncytial giant cells expressed immunoreactivity (Fig. 2), indicating that some of them had been dead and/or had released most of the viral antigens. The immunoreactivity would then have decreased to an undetectable level.

The titers of RCVs are summarized in Table 2, and expressed as the reciprocal of the highest dilution forming the foci (focus-forming unit (FFU)/m*l*). The original viruses were low to intermediate infectivity titers, as was the RCV/LBC/0 fluid (data not shown). It has been reported that RCV were not propagated in LBC cells [4]. Although we did not observe CPE in LBC cells (Fig. 1a), slight growth of both RCVs was noted in these cells. This result may have been due to the incubation of the LBC cells at a low temperature (33°C).

In the virus suspensions of the first and second RCN-9 passage (RCV/RCN-9/1, 2), the infectivity titers assayed in both LBC and RCN-9 cells were higher than the initial RCV/LBC/0. The log ratio of the infectivity titer in RCN-9 cells to that in LBC cells was 0.6–2.3 through LBC cells and 2.5–3.9 through RCN-9 cells. Both RCVs replicated in LBC cells show higher tropism toward RCN-9 cells than to LBC cells, suggesting that RCN-9 cells are more suitable for the replication of RCV than LBC cells. In RCN-9 cells, similar susceptibility to the mouse L-2 cells [11, 12] was observed

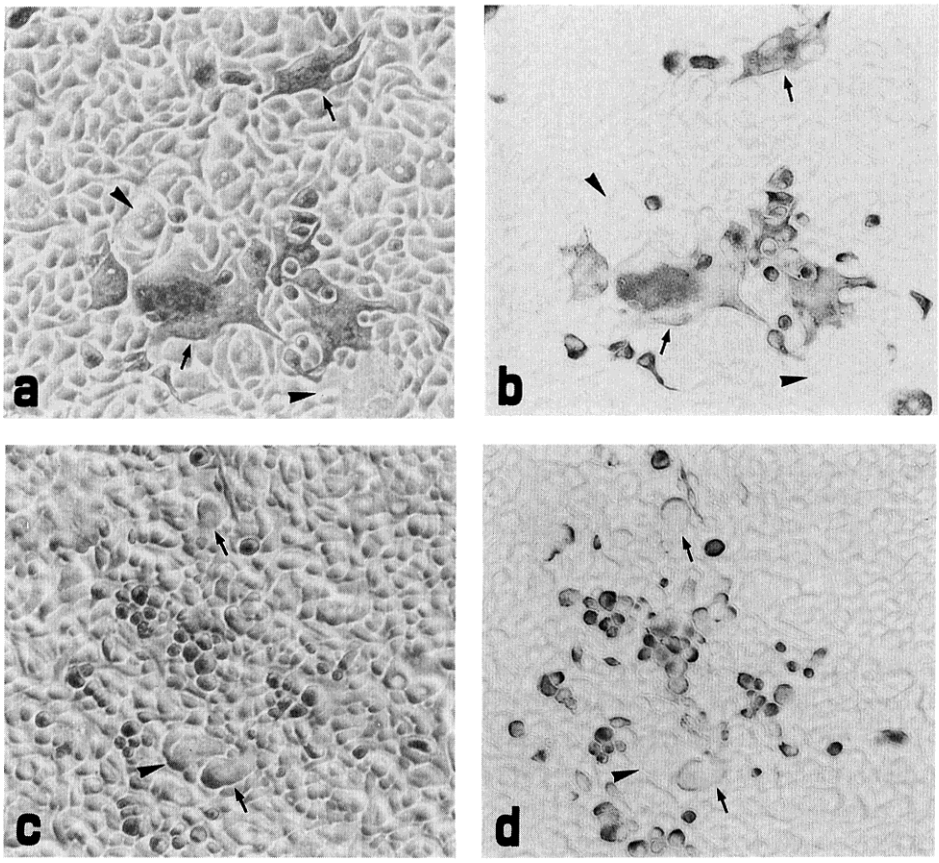

Fig. 2. Interference micrograph of RCN-9 cells infected with the SDAV-681/RCN-9/1 of $1:10^4$ dilution (a) and PRCV/RCN-9/1 (c) on 2 dpi. Syncytial giant cells (arrows) are shown with immunoreactivity, but not all of the giant cells express immunoreactivity (arrow heads). (b) and (d) are micrographs of the same field of vision shown in Figures 2a and 2c, respectively. The syncytial giant cells of SDAV-681 infected RCN-9 are larger than those of PRCV. × 160.

at 37°C in 4 dpi. RCN-9 cells are 1, 2-dimethylhydrazine-induced tumor cells derived from the colon of an F344 male rat [8]. The cells characteristically metastasize to the lungs (7/11; 64%) and the liver (2/5; 40%), after being injected subcutaneously and into cecal subserous tissues, respectively. We found that almost all of the RCN-9 cells were anti-keratin-positive (ABC method, employing rabbit anti-keratin wide-spectrum screening antibody; Daco Co., CA, U.S.A.), indicating an epithelial cell origin. RCN-9 cells may make a useful tool in analyzing the relation between RCVs infections and epithelial tumor cells of digestive tracts.

It has been shown that experimental intranasal inoculation [17] and implanted cells [5] induced persistent RCV infections in athymic rats. In raising immunodeficient mice and rats, we need to consider more carefully RCV infections, as well as MHV infection. The RCN-9 cell may be a helpful tool with which to reveal differences in the tissue tropism and genetic properties of RCV strains, and it may be useful for illuminating the mechanism of RCV infections both *in vitro* and *in vivo*.

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